

Atty Dkt No. 8325-0002.221  
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PATENT

**REMARKS**

The foregoing amendments are made to insert the sequence identification numbers into the specification.

Respectfully submitted,

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Version with markings to show changes made

In the specification:

Paragraph beginning on page 3, line 7, has been amended as follows:

Zinc finger proteins (“ZFPs”) are proteins that bind to DNA, RNA and/or protein in a sequence-specific manner. Zinc fingers were first identified in the transcription factor TFIIIA from the oocytes of the African clawed toad, *Xenopus laevis*. ZFPs are widespread in eukaryotic cells. An exemplary motif characterizing one class of these proteins (C<sub>2</sub>H<sub>2</sub> class) is -Cys-(X)<sub>2-4</sub>-Cys-(X)<sub>12</sub>-His-(X)<sub>3-5</sub>-His (SEQ ID NO:1) (where X is any amino acid). A single finger domain is about 30 amino acids in length and several structural studies have demonstrated that it contains an alpha helix containing the two invariant histidine residues co-ordinated through zinc with the two cysteines of a single beta turn. To date, over 10,000 zinc finger sequences have been identified in several thousand known or putative transcription factors. ZFPs are involved not only in DNA-recognition, but also in RNA binding and protein-protein binding. Current estimates are that this class of molecules will constitute about 2% of all human genes.

Paragraph beginning on page 15, line 14, has been amended as follows:

A “zinc finger binding protein” is a protein or polypeptide that binds DNA, RNA and/or protein, preferably in a sequence-specific manner, as a result of stabilization of protein structure through coordination of a zinc ion. The term zinc finger binding protein is often abbreviated as zinc finger protein or ZFP. The individual DNA binding domains are typically referred to as “fingers”. A ZFP has least one finger, typically two fingers, three fingers, or six fingers. Each finger binds from two to four base pairs of DNA, typically three or four base pairs of DNA. A ZFP binds to a nucleic acid sequence called a target site or target segment. Each finger typically comprises an approximately 30 amino acid, zinc-chelating, DNA-binding subdomain. An exemplary motif characterizing

one class of these proteins ( $C_2H_2$  class) is -Cys-(X)<sub>2-4</sub>-Cys-(X)<sub>12</sub>-His-(X)<sub>3-5</sub>-His (SEQ ID NO:1) (where X is any amino acid). Studies have demonstrated that a single zinc finger of this class consists of an alpha helix containing the two invariant histidine residues coordinated with zinc along with the two cysteine residues of a single beta turn (see, e.g., Berg & Shi, *Science* 271:1081-1085 (1996)).

Paragraph beginning on page 30, line 12, has been amended as follows:

A D-able site or subsite is a region of a target site that allows an appropriately designed single zinc finger to bind to four bases rather than three of the target site. Such a zinc finger binds to a triplet of bases on one strand of a double-stranded target segment (target strand) and a fourth base on the other strand (see Figure 2 of co-owned PCT WO 00/42219). Binding of a single zinc finger to a four base target segment imposes constraints both on the sequence of the target strand and on the amino acid sequence of the zinc finger. The target site within the target strand should include the "D-able" site motif 5' NNGK 3' (SEQ ID NO:41), in which N and K are conventional IUPAC-IUB ambiguity codes. A zinc finger for binding to such a site should include an arginine residue at position -1 and an aspartic acid, (or less preferably a glutamic acid) at position +2. The arginine residues at position -1 interacts with the G residue in the D-able site. The aspartic acid (or glutamic acid) residue at position +2 of the zinc finger interacts with the opposite strand base complementary to the K base in the D-able site. It is the interaction between aspartic acid (symbol D) and the opposite strand base (fourth base) that confers the name D-able site. As is apparent from the D-able site formula, there are two subtypes of D-able sites: 5' NN<sub>G</sub>G 3' (SEQ ID NO:42) and 5' NN<sub>G</sub>T 3' (SEQ ID NO:43). For the former site, the aspartic acid or glutamic acid at position +2 of a zinc finger interacts with a C in the opposite strand to the D-able site. In the latter site, the aspartic acid or glutamic acid at position +2 of a zinc finger interacts with an A in the

opposite strand to the D-able site. In general, NNGG (SEQ ID NO:42) is preferred over NNGT (SEQ ID NO:43).

Paragraph beginning on page 31, line 16, has been amended as follows:

In the formula 5'-NNx aNy bNzc-3', the triplets of NNx aNy and bNzc represent the triplets of bases on the target strand bound by the three fingers in a ZFP. If only one of x, y and z is a G, and this G is followed by a K, the target site includes a single D-able subsite. For example, if only x is G, and a is K, the site reads 5'-**NNG** KNy bNzc-3' with the D-able subsite highlighted. If both x and y but not z are G, and a and b are K, then the target site has two overlapping D-able subsites as follows: 5'-**NNG** *KNG* KNz c-3' (SEQ ID NO:2), with one such site being represented in bold and the other in italics. If all three of x, y and z are G and a, b, and c are K, then the target segment includes three D-able subsites, as follows 5'**NNG** *KNG* KNG K3' (SEQ ID NO:3), the D-able subsites being represented by bold, italics and underline.

Paragraph beginning on page 49, line 3, has been amended as follows:

Linker domains between polypeptide domains, e.g., between two ZFPs or between a ZFP and a regulatory domain, can be included. Such linkers are typically polypeptide sequences, such as poly gly sequences of between about 5 and 200 amino acids. Preferred linkers are typically flexible amino acid subsequences which are synthesized as part of a recombinant fusion protein. For example, in one embodiment, the linker DGGGS (SEQ ID NO:4) is used to link two ZFPs. In another embodiment, the flexible linker linking two ZFPs is an amino acid subsequence comprising the sequence TGEKP (SEQ ID NO:5) (see, e.g., Liu *et al.*, PNAS 5525-5530 (1997)). In another embodiment, the linker LRQKDGERP (SEQ ID NO:6) is used to link two ZFPs. In another embodiment, the following linkers are used to link two ZFPs: GGRR (SEQ ID NO:7) (Pomerantz *et al.* 1995, *supra*), (G4S)<sub>n</sub> (SEQ ID NO:8) (Kim *et al.*, PNAS 93,

1156-1160 (1996.); and GGRRGGGS (SEQ ID NO:9); LRQRDGERP (SEQ ID NO:10); LRQKDGGGSERP (SEQ ID NO:11); LRQKd(G3S)<sub>2</sub> ERP (SEQ ID NO:12).

Alternatively, flexible linkers can be rationally designed using computer program capable of modeling both DNA-binding sites and the peptides themselves (Desjarlais & Berg, *PNAS* 90:2256-2260 (1993), *PNAS* 91:11099-11103 (1994) or by phage display methods.

Paragraph beginning on page 74, line 24, has been amended as follows:

This first Example demonstrates the construction of ZFPs designed to recognize DNA sequences contained in the promoter of the human vascular endothelial growth factor (VEGF) gene. VEGF is an approximately 46 kDa glycoprotein that is an endothelial cell-specific mitogen induced by hypoxia. VEGF has been implicated in angiogenesis associated with cancer, various retinopathies, and other serious diseases. The DNA target site chosen was a region surrounding the transcription initiation site of the gene. The two 9 base pair (bp) sites chosen are found within the sequence agcGGGGAGGATcGCGGAGGCTtgg (SEQ ID NO:13), where the upper-case letters represent actual 9-bp targets. The protein targeting the upstream 9-bp target was denoted VEGF1, and the protein targeting the downstream 9-bp target was denoted VEGF3a. The major start site of transcription for VEGF is at the T at the 3' end of the first 9-bp target, which is underlined in the sequence above.

Paragraph beginning on page 76, line 10, has been amended as follows:

VEGF1:

GGTACCCATACTGGCAAGAAGAACAGCACATCTGCCACATCCAGGGCTGT  
GGTAAAGTTACGGCACAAACCTCAAATCTGCGTCGTCACCTGCGCTGGCACA  
CCGGCGAGAGGCCTTCATGTGTACCTGGCCTACTGTGGTAAACGCTTCACC  
CGTCGTCAAACCTGCAGCGTCACAAGCGTACCCACACCGGTGAGAAGAAAT

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TTGCTTCCCCGGAGTGTCCGAAGCGCTTCATGCGTAGTGACCACCTGTCCCGT  
CACATCAAGACCCACCAGAATAAGAAGGGTGGATCC (SEQ ID NO:14)

Paragraph beginning on page 76, line 17, has been amended as follows:

VEGF1 translation:

VPIPGKKQHICHIQCGKVYGTTSNLRRHLRWHTGERPFMCTWSYCGKRFTRS  
SNLQRHKRTHTGEKKFACPECPKRFMRSDHLSRHIKTHQNKKGGS (SEQ ID  
NO:15)

Paragraph beginning on page 76, line 20, has been amended as follows:

VEGF3a:

GGTACCCATACCTGGCAAGAAGAACAGCACATCTGCCACATCCAGGGCTGT  
GGTAAAGTTACGGCCAGTCCTCCGACCTGCAGCGTCACCTGCGCTGGCACA  
CCGGCGAGAGGCCTTCATGTGTACCTGGTCCTACTGTGGTAAACGCTTCACC  
CGTCGTCAAACCTACAGAGGCACAAGCGTACACACACCCGGTGAGAAGAAAT  
TTGCTTCCCCGGAGTGTCCGAAGCGCTTCATGCGAAGTGACGAGCTGTCACG  
ACATATCAAGACCCACCAGAACAAAGAAGGGTGGATCC (SEQ ID NO:16)

Paragraph beginning on page 76, line 27, has been amended as follows:

VEGF3a translation:

VPIPGKKQHICHIQCGKVYQSSDLQRHLRWHTGERPFMCTWSYCGKRFTRS  
SNLQRHKRTHTGEKKFACPECPKRFMRSDLSRHIKTHQNKKGGS (SEQ ID  
NO:17)

Paragraph beginning on page 78, line 14, has been amended as follows:

VEGF site 1, top: 5'-CATGCATAGCGGGGAGGGATGCCATCGAT (SEQ ID NO:18)

VEGF site 1, bottom: 5'-ATCGATGGCGATCCTCCCCGCTATGCATG (SEQ ID NO:19)

VEGF site 3, top: 5'-CATGCATATCGCGGAGGGCTTGGCATCGAT (SEQ ID NO:20)

VEGF site 3, bottom: 5'-ATCGATGCCAAGCCTCCGCGATATGCATG (SEQ ID NO:21)

Paragraph beginning on page 80, line 2, has been amended as follows:

An important consideration in ZFP design is DNA target length. For random DNA, a sequence of n nucleotides would be expected to occur once every  $0.5 \times 4^n$  base-pairs. Thus, DNA-binding domains designed to recognize only 9 bp of DNA would find sites every 130,000 bp and could therefore bind to multiple locations in a complex genome (on the order of 20,000 sites in the human genome). 9-bp putative repressor-binding sequences have been chosen for VEGF in the 5' UTR where they might directly interfere with transcription. However, in case zinc finger domains that recognize 9-bp sites lack the necessary affinity or specificity when expressed inside cells, a larger domain was constructed to recognize 18 base-pairs by joining separate three-finger domains with a linker sequence to form a six-finger protein. This should ensure that the repressor specifically targets the appropriate sequence, particularly under conditions where only small amounts of the repressor are being produced. The 9-bp target sites in VEGF were chosen to be adjacent to one another so that the zinc fingers could be linked to recognize an 18-bp sequence. The linker DGGGS (SEQ ID NO:4) was chosen because it permits binding of ZFPs to two 9-bp sites that are separated by a one nucleotide gap, as is the case for the VEGF1 and VEGF3a sites (*see also* Liu *et al.*, PNAS 5525-5530 (1997)).

Paragraph beginning on page 80, line 18, has been amended as follows:

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The 6-finger VEGF3a/1 protein encoding sequence was generated as follows. VEGF3a was PCR amplified using the primers SPE7 (5'-  
GAGCAGAATTCGGCAAGAAGAACGCAC (SEQ ID NO:22) and SPEamp12 (5'-  
GTGGTCTAGACAGCTCGTCACTTCGC (SEQ ID NO:23) to generate EcoRI and XbaI restriction sites at the ends (restriction sites underlined). VEGF1 was PCR amplified using the primers SPEamp13 (5'-  
GGAGCCAAGGCTGTGGTAAAGTTACGG (SEQ ID NO:24) and SPEamp11 (5'-  
GGAGAAGCTGGATCCTCATTATCCC (SEQ ID NO:25) to generate StyI and HindIII restriction sites at the ends (restriction sites underlined). Using synthetic oligonucleotides, the following sequence was ligated between the XbaI and StyI sites, where XbaI and StyI are underlined: TCT AGA CAC ATC AAA ACC CAC CAG AAC AAG AAA GAC GGC GGT GGC AGC GGC AAA AAG AAA CAG CAC ATA TGT CAC ATC CAA GG (SEQ ID NO:26). This introduced the linker sequence DGGGS (SEQ ID NO:4) between the two SP-1 domains. The ligation product was reamplified with primers SPE7 and SPEamp11 and cloned into pUC19 using the EcoRI and HindIII sites. The linked ZFP sequences were then amplified with primers

(1) GB19

GCCATGCCGGTACCCATACCCTGGCAAGAAGAACGCAC (SEQ ID NO:27)

(2) GB10

CAGATCGGATCCACCCTCTTATTCTGGTGGGT (SEQ ID NO:28) to introduce KpnI and BamHI sites for cloning into the modified pMAL-c2 expression vector as described above.

Paragraph beginning on page 81, line 8 has been amended as follows:

The nucleotide sequence of the designed, 6-finger ZFP VEGF3a/1 from KpnI to BamHI is:

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GGTACCCATACCTGGCAAGAAGAACGAGCACATCTGCCACATCCAGGGCTGT  
GGTAAAGTTACGGCCAGTCCTCCGACCTGCAGCGTCACCTGCGCTGGCACA  
CCGGCGAGAGGCCTTCATGTGTACCTGGTACTGTGGTAAACGCTTCACA  
CGTCGTCAAACCTACAGAGGCACAAGCGTACACACACAGGTGAGAAGAAA  
TTTGCTTCCCCGGAGTGTCCGAAGCGCTTCATGCGAAGTGACGAGCTGTCTAG  
ACACATCAAAACCCACCAGAACAAAGAACGCGGTGGCAGCGGCAAAAAA  
GAAACAGCACATATGTCACATCCAAGGCTGTGGTAAAGTTACGGCACAACC  
TCAAATCTGCGTCGTACCTGCGCTGGCACACCGCGAGAGGCCTTCATGTG  
TACCTGGTCCTACTGTGGTAAACGCTTCACCGTTCGTCAAACCTGCAGCGTC  
ACAAGCGTACCCACACCGGTGAGAAGAAATTGCTGCCGGAGTGTCCGAA  
GCGCTTCATGCGTAGTGACCACCTGTCCGTACATCAAGACCCACCAGAAT  
AAGAAGGGTGGATCC (SEQ ID NO:29)

Paragraph beginning on page 81, line 22, has been amended as follows:

The VEGF3a/1 amino acid translation (using single letter code) is:

VPIPGKKQHICHIQGCGKVYVGQSSDLQRHLRWHTGERPFMCTWSYCGKRFTRS  
SNLQRHKRTHGEKKFACPECPKRFMRSDELSRHIKTHQNKKDGGSGKKQHI  
CHIQGCGKVYGTTSNLRRHLRWHTGERPFMCTWSYCGKRFRSSNLQRHKRTH  
TGEKKFACPECPKRFMRSDHLSRHIKTHQNKKGGS (SEQ ID NO:30)

Paragraph beginning on page 81, line 27, has been amended as follows:

The 18-bp binding protein VEGF3a/1 was expressed in *E. coli* as an MBP fusion, purified by affinity chromatography, and tested in EMSA experiments as described in Example 1. The target oligonucleotides were prepared as described and comprised the following complementary sequences:

(1) JVF9

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AGCGAGCGGGGAGGATCGCGGAGGCTGGGGCAGCCGGTAG (SEQ ID NO:31),  
and

(2) JVFI0

CGCTCTACCCGGCTGCCCAAGCCTCCGCATCCTCCCCGCT (SEQ ID NO:32).

Paragraph beginning on page 83, line 22, has been amended as follows:

The VP16 protein of HSV-1 has been studied extensively, and it has been shown that the C-terminal 78 amino acids can act as a trans-activation domain when fused to a DNA-binding domain (Hagmann *et al.*, *J. Virology* 71:5952-5962 (1997)). VP16 has also been shown to function at a distance and in an orientation-independent manner. For these studies, amino acids 413 to 490 in the VP16 protein sequence were used. DNA encoding this domain was PCR amplified from plasmid pMSVP16ΔC+119 using primers with the following sequences:

(1) JVFI4

CGCGGATCCGCCCCCCCACCGATG (SEQ ID NO:33), and

(2) JVFI5

CCGCAAGCTTACTTGTCAATCGTCGTCTTGATTCGCTGCCACCCTACTC  
GTCAATTCC (SEQ ID NO:34).

Paragraph beginning on page 84, line 5, has been amended as follows:

Three expression vectors were constructed for these studies. The general design is summarized in Figure 5. The vectors are derived from pcDNA3.1(+) (Invitrogen), and place the ZFP constructs under the control of the cytomegalovirus (CMV) promoter. The vector carries ampicillin and neomycin markers for selection in bacteria and mammalian cell culture, respectively. A Kozak sequence for proper translation initiation (Kozak, *J. Biol. Chem.* 266:19867-19870 (1991)) was incorporated. To achieve nuclear localization of the products, the nuclear localization sequence (NLS) from the SV40 large T antigen (Pro-Lys-

Lys-Lys-Arg-Lys-Val (SEQ ID NO:35) (Kalderon *et al.*, *Cell* 39:499-509 (1984)) was added. The insertion site for the ZFP-encoding sequence is followed by the functional domain sequence. The three versions of this vector differ in the functional domain; “pcDNA-NKF” carries the KRAB repression domain sequence, “pcDNA-NVF” carries the VP16 activation domain, and “NF-control” carries no functional domain. Following the functional domain is the FLAG epitope sequence (Kodak) to allow specific detection of the ZFPs.

Paragraph beginning on page 84, line 19, has been amended as follows:

The vectors were constructed as follows. Plasmid pcDNA-ΔHB was constructed by digesting plasmid pcDNA3.1(+) (Invitrogen) with HindIII and BamHI, filling in the sticky ends with Klenow, and religating. This eliminated the HindIII, KpnI, and BamHI sites in the polylinker. The vector pcDNA3.1(+) is described in the Invitrogen catalog. Plasmid pcDNA-NKF was generated by inserting a fragment into the EcoRI/XhoI sites of pcDNA-ΔHB that contained the following: 1) a segment from EcoRI to KpnI containing the Kozak sequence including the initiation codon and the SV40 NLS sequence, altogether comprising the DNA sequence

GAATTCGCTAGCGCCACCATGGCCCCAAGAAGAAGAGGAAGGTGGAAATC  
CATGGGGTAC (SEQ ID NO:36),

where the EcoRI and KpnI sites are underlined; and 2) a segment from KpnI to XhoI containing a BamHI site, the KRAB-A box from KOX1 (amino acid coordinates 11-53 in Thiesen, 1990, *supra*), the FLAG epitope (from Kodak/IBI catalog), and a HindIII site, altogether comprising the sequence

GGTACCCGGGGATCCCGGACACTGGTGACCTCAAGGATGTATTGTGGACT  
TCACCAGGGAGGAGTGGAAAGCTGCTGGACACTGCTCAGCAGATCGTGTACAG  
AAATGTGATGCTGGAGAACTATAAGAACCTGGTTCCCTGGGCAGCGACTAC  
AAGGACGACGATGACAAGTAAGCTTCTCGAG (SEQ ID NO:37)

where the KpnI, BamHI and XhoI sites are underlined.

Paragraph beginning on page 85, line 15, has been amended as follows:

The effector plasmids used in Example 5 were constructed as follows. Plasmid pcDNA-NVF was constructed by PCR amplifying the VP16 transactivation domain, as described above, and inserting the product into the BamHI/HindIII sites of pcDNA-NKF, replacing the KRAB sequence. The sequence of the inserted fragment, from BamHI to HindIII, was:

GGATCCGCCCCCGACCGATGTCAGCCTGGGGACGAGCTCCACTAGACG  
GCGAGGACGTGGCGATGGCGATGCCGACCGCTAGACGATTGATCTGGA  
CATGTTGGGGACGGGATTCCCCGGGCCGGATTACCCCCACGACTCC  
GCCCTACGGCGCTCTGGATATGGCGACTTCGAGTTGAGCAGATGTTAC  
CGATGCCCTTGAATTGACGAGTACGGTGGGGCAGCGACTACAAGGACGAC  
GATGACAAGTAAGCTT (SEQ ID NO:38).

Paragraph beginning on page 85, line 29, has been amended as follows:

The effector plasmids used in Example 6 were constructed as follows. Plasmid NF-control was generated by inserting the sequence

GAATTCGCTAGCGCCACCATGGCCCCAAGAAGAAGAGGAAGGTGGAAATC  
CATGGGGTACCCGGGGATGGATCCGGCAGCGACTACAAGGACGACGATGAC  
AAGTAAGCTTCTCGAG (SEQ ID NO:39)

into the EcoRI-XhoI sites of pcDNA-NKF, thereby replacing the NLS-KRAB-FLAG sequences with NLS-FLAG only.

Paragraph beginning on page 87, line 3, has been amended as follows:

The reporter plasmid system was based on the pGL3 firefly luciferase vectors (Promega). Four copies of the VEGF target sites were inserted upstream of the SV40

promoter, which is driving the firefly luciferase gene, in the plasmid pGL3-Control to create pVFR1-4x. This plasmid contains the SV40 enhancer and expresses firefly luciferase to high levels in many cell types. Insertions were made by ligating together tandem copies of the two complementary 42-bp oligonucleotides, JV9 and JV10, described in Example 2. Adaptor sequences were ligated on, and the assembly was inserted into the MluI/BglII sites of pGL3-Control. This resulted in the insertion of the following sequence between those sites:

ACGCGTaagcttGCTAGCGAGCGGGGAGGATCGCGGAGGCTGGGCAGCCGGG  
TAGAGCGAGCGGGGAGGATCGCGGAGGCTGGGCAGCCGGTAGAGCGAGCCGGGGAGGA  
TCGCGGAGGCTGGGCAGCCGGTAGAGCGCTCAgaagcttAGATCT (SEQ ID NO:40).

Paragraph beginning on page 98, line 21, has been amended as follows:  
HEK 293 cells were seeded to a concentration of  $0.5 \times 10^6$  cells per well in 6-well plates. One day later, the cells were transfected (2 ug plasmid DNA in 7 ul LipofectAMINE 2000) with a plasmid encoding a ZFP-functional domain fusion. The ZFP binding domain used in the fusions, Cat18a, was designed (as described *supra* and in WO 98/53059; WO 98/53059; WO 98/53060 and co-owned WO 00 42219) to bind to a 9-nucleotide sequence (GTGGGGGGCl, SEQ ID NO: \_\_\_\_ ]) located between 75 and 83 nucleotides upstream of the transcription startsite disclosed by Hara *et al.* (1996) *Mol. Cell. Biol.* **16**:859-867. Sequences encoding this binding domain were fused independently to sequences encoding the KRAB (Example 3, *supra*), v erbA (amino acids 223-556), and MBD2B (amino acids 149-411) repression domains.